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INTRODUCTION

Apoptosis is a programmed form of cell death that plays an important role in malignancy by shifting the balance from tumor proliferation to its regression. Anticancer drugs act by activating apoptosis in tumor cells. Mutations in apoptotic pathways can lead to anticancer drug resistance and therefore can promote tumor progression. Our lab is working to elucidate the molecular mechanisms of apoptosis in oncogenically-transformed primary Mouse Embryo Fibroblasts (MEFs). We have chosen this model system because it lacks mutations and alterations that are common to immortal cell lines; of the ability to use genetic approach to study apoptosis (availability of knock-out mouse lines); of the ease of gene manipulations (retroviral mediated gene transfer technique) in MEFs. The adenovirus E1A oncoprotein sensitizes primary cells to undergo apoptosis following treatment with anticancer agents. It was shown that E1A induced sensitivity in MEFs is similar to chemosensitivity of spontaneous tumors. We expect that further insight into mechanisms of programmed cell death in oncogenically-transformed MEFs will provide a fuller understanding of the role of apoptosis in real tumor progression such as breast cancer and will lead to the developing new strategies for anti-cancer therapy.

I am interested in determining the role of caspases in apoptosis in MEFs. Caspases are a family of cystein proteases that are expressed in latent pro-enzyme forms. Caspases are essential components of apoptotic machinery, and activation of pro-caspases is an obligatory step in the induction and execution of apoptosis triggered by many anticancer drugs. The goal of my thesis project is to identify caspases that are involved in programmed cell death in primary MEFs, to reveal caspases activated during distinct apoptotic programs, to determine the effect of E1A expression on caspases in the cells and finally to establish the role of one particular caspase in distinct forms of apoptosis in oncogenically-transformed MEFs.

BODY

Aim 1. Identification and characterization of distinct apoptotic pathways in E1A/ras-transformed MEFs.

Aim 1 was completed during previous years.

Aim 2. Identification of caspases activated during distinct apoptotic programs in ER/MEFs.

Aim 2 was completed during previous years.

Aim 3. Establishing the role of Caspase-2 in distinct forms of apoptosis in oncogenically transformed mouse embryo fibroblasts.

Aim 3 was completed during previous years.

Aim 4. Determination of the mechanism by which the E1A oncogene induces the pro-caspase levels.

ABSTRACT

The adenovirus E1A oncogene promotes apoptosis through p53-dependent and independent pathways. Here we show that E1A induces several pro-caspases through a p53-independent mechanism that involves inactivation of Rb and deregulation of E2F. Hence, E1A induces several caspases in human and murine fibroblasts in a manner strictly dependent on the E1A-Rb interaction. This effect can be recapitulated by overexpression of E2F-1, and occurs in ARF-null and p53-null cells. Rb-null fibroblasts displayed constitutively elevated levels of pro-caspase proteins, and re-introduction of Rb into human tumor cell deficient in Rb suppressed caspase levels in a manner dependent on the Rb pocket. Pro-caspase induction does not involve increased transcription or protein stability, but rather involves enhanced translation of pro-caspase mRNAs. This unexpected finding demonstrates that pro-caspases can be controlled at the translational level, and suggests that this contribute to the pro-apoptotic activity of E1A, Rb loss, and E2F.

INTRODUCTION

Apoptosis and cell proliferation are two major counteracting processes balance between, which is crucial for normal development and maintaining homeostasis of the tissues in adult. Cancer results when group of mutated cells survive and begin to proliferate inappropriately, shifting this balance. Tumorigenic mutations cause activation of cellular oncogenes, which induce uncontrolled proliferation. However, deregulation of these genes often results in induction of apoptosis or increased sensitivity to a variety of apoptosis inducing agents (Evan and Littlewood 1998). Mutations disabling apoptosis allows oncogenes induce only proliferation and this facilitates transformation.

Oncogenes may promote apoptosis either indirectly as a consequence of forced entry into cell cycle or through activation of parallel apoptotic pathway. The mechanisms of oncogene-induced apoptosis and sensitivity are poorly defined yet. It is known that E1A oncogene promote apoptosis, in part, through binding and inactivation of the retinoblastoma (Rb) protein (Samuelson and Lowe 1997) and stabilization of p53 tumor suppressor through ARF another tumor suppressor encoded by INK4a/ARF locus (Bates et al. 1998; de Stanchina et al. 1998). One consequence of Rb inactivation is derepression of E2F family members, resulting in the transcription of genes required for S-phase (Dyson 1998). Loss of function of Rb is thus associated with increased proliferation; however, inactivation of Rb can also trigger apoptosis (Adams and Kaelin 1998).

E2F-1 plays an important role to limit the oncogenic consequences of Rb loss by promoting apoptosis through activation of p53 dependent (p19ARF-p53) (Qin et al. 1994) and p53 independent pathways (Qin et al. 1994; Hsieh et al. 1997; Phillips et al. 1997). E2F-1 was shown to directly transcriptionally activate the $p19^{ARF}$ gene (Bates et al. 1998).

Apoptosis triggered by induction of oncogenes or by anti-cancer agents ultimately leads to activation of caspases, a family of cysteine proteases that are essential components of the cell death machinery. Caspases are expressed as latent pro-enzymes and processed to active enzymes during early stages of apoptotic cell death by another protease or by autocatalysis. Thus, large amount of precursor can be accumulated in advance. Caspases can be divided into two major groups: one with long pro-domain (caspases 2, 8, 9, 10) called regulatory or initiator caspases. They believes to act at the early stages of caspases cascade as they serve as an acceptors and propagators of apoptotic signals. Second group - caspases with short pro-domain (caspase 3, 6, 7) called executioner caspases that predominantly function in the bottom of caspase cascade and which role is to cleave theirs specific target proteins, resulting in cell disassembly (Kumar 1999). There are two well-described pathways of caspase activation. One is triggered by binding of the ligand to a death receptor, which leads to recruitment of pro-caspase 8 into a multiprotein complex where autoactivation and transactivation occurs. In the second pathway variety of death stimuli cause the release of cytochrome c from mitochondria. Cytochrome c binds to APAF1 and in the presence of ATP, recruits pro-caspase 9 to the apoptosome complex where activation of pro-caspase 9 occurs (Budihardjo et al. 1999). Recently, a several lines of evidence implicated APAF1-caspase 9 pathway in mediating oncogene-dependent death signaling. Mutations that disable this pathway can facilitate oncogenic transformation (Fearnhead et al. 1998; Soengas et al. 1999). c-myc–induced apoptosis may involve components related to Fas death receptor pathway (Hueber et al. 1997). Control of cytochrome c release is another major site of death regulation by oncogenes. Overexpression of c-myc causes cytochrome c release predisposing cells to apoptosis triggered by different death stimuli (Juin et al. 1999). In contrast E1A expression does not result in cytochrome c release directly, but sensitize cells to other death stimuli that then lead to cytochrome c release (Fearnhead et al. 1998).

Proteolytic processing is a major form of the regulation of caspase activity. Little is known about the mechanism of this regulation. Although recent studies provided indication that the control of caspase activation is achieved both at the level of caspase recruitment and pro-caspase oligomerisation (Budihardjo et al. 1999; Kumar 1999). According to the induced-proximity model (Salvesen and Dixit 1999) clustering of pro-enzymes (pro-caspases) possessing intrinsic enzymatic activity would ultimately lead to auto processing and activation of the first protease in the cascade. The increased expression of pro-caspases genes would lead to increase in local concentration and presumably facilitate oligomerization and processing. It was shown that overexpression of caspases precursors in different cell type induce apoptosis (Miura et al. 1993; Fernandes-

Alnemri et al. 1994; Kumar et al. 1994; Wang et al. 1994; Boldin et al. 1996; Srinivasula et al. 1996). Hence, upregulation of caspase genes expression can be one of the mechanisms for caspase regulation. No experimental data exist on this subject, except the study that showed that caspase 2 and 3 genes were induced in human leukemic cell lines following treatment with etoposide (Droin et al. 1998). A few lines of evidence suggest that phosphorylation can be another form of regulation. Recently it was shown that phosphorylation of caspase 9 on serine-196 inhibited its protease activity in vitro (Cardone et al. 1998).

In this study, we examine how caspases are regulated in response to oncogenes. We used E1A as a tool to dissect cellular processes involved in caspase regulation. To this end, we found that E1A greatly increase the levels of pro-caspases through mechanism that involves the ability of E1A to influence the translational efficiency of caspases mRNAs. Consequently, we genetically demonstrated that this function of E1A involves its ability to deregulate the Rb pathway. These studies provide some of the first insights into control of pro-caspase levels, and provide a link between cell-cycle control and the regulation of the apoptotic machinery.

RESULTS

E1A Oncogene Induces Pro-caspase Levels.

To determine how E1A promote apoptosis we examine the influence of E1A expression on the status of apoptotic machinery in the cell. Pro-caspase 1,2,3,7,8 and 9 expression was analyzed in primary MEFs and in human normal diploid lung fibroblasts IMR90. Cells infected with E1A oncogene expressed significally more pro-caspases than cells infected with control vector (Fig. 1). On average, pro-caspase levels in E1A-expressing cells were 5-10 fold higher than in control cells. Since E1A promotes chemosensitivity both in human and mouse fibroblasts by a variety of agents (Lowe et al. 1993) through inactivation of Rb protein (Samuelson and Lowe 1997) and induction of p53 through ARF (Bates et al. 1998; de Stanchina et al. 1998), induction of pro-caspase levels by E1A might synergize with these mechanisms.

CR2 Region of E1A is Required for Pro-caspase Induction.

To determine whether the same regions of E1A that are responsible for chemosensitivity and p53 induction are required for pro-caspase induction, two deletion E1A mutants were used - ΔN (deletion of amino-acids 2-36) and ΔCR2 (deletion of amino acids 120-140) (Samuelson and Lowe 1997). These mutants were compromised in their ability to physically interact with either the p300/p400 (ΔN) or Rb/p107/p130 (ΔCR2) (Wang et al. 1993). We examine the ability of these E1A mutants to induce pro-caspases in MEFs and IMR90 (Fig. 2). Cells expressing full-length E1A displayed dramatic increase in pro-caspase 3 and 7 levels. The ΔCR2 mutant was defective in induction of both pro-caspase 3 and 7. Contrary to that ΔN mutant was able to induce pro-caspase 3 levels but was

much less efficient than full length E1A in altering pro-caspase 7 levels both in MEFs and in IMR90. From now on we decided to concentrate on these two major execution caspases 3 and 7 because of differences in regulation by E1A and availability of good antibodies for both mouse and human proteins. Thus, the same E1A functions that promote apoptosis and induce p53 are required for pro-caspase induction.

E1A-mediated Pro-caspase Induction Occurs at the Level of Protein Translation.

Cells expressing E1A accumulate p53 protein due, in part, to increased p53 stability (Lowe and Ruley 1993). To determine the mechanism whereby pro-caspase levels were increased levels of pro-caspase gene transcripts, rate of protein turnover and translation efficiency rate were measured.

Northern blot analysis was performed to establish the regulation on the level of mRNA expression (Fig.3A). Amount of mRNA specific to caspase 2, 3, 6, 7, 8 were practically the same in E1A-expressing MEFs compared to control MEFs pointing towards post-transcriptional mechanism of induction.

Proteosome plays an important role in degradation of p53 (Maki et al. 1996). To access the role of proteosome proteolysis in regulation of pro-caspase turnover primary MEFs were treated with two known proteosome inhibitors – peptide aldehydes (LLnL and MG132). As a control calpain 2 inhibitor LLM that is incapable of inhibiting proteosome were used (Fig. 3B). LLnL and MG132 caused dramatic accumulation of p53 protein in concordance with previous results (Maki et al. 1996). However, levels of pro-caspase 3 and 7 decreased with time. That was due to the toxicity of proteosome inhibitors to the cells, since at time points 4 to 24 hours after start of the treatment cells began to die by apoptosis. Processing of caspase 7 is the evidence of that. Thus, protein levels of pro-caspases are not regulated by proteosome.

To examine pro-caspase turnover, IMR90 expressing E1A and control vector were labeled with [^{35}S]methionine for 1 hour and chased with excess unlabeled methionine for various times. Levels of labeled pro-caspase 3 and 7 were monitored by immunoprecipitation. Autoradiograghs illustrating pro-caspase 3 and 7 turnover in IMR90 expressing vector or E1A are shown in Figure 3C. The half-life of p53 is 20-30 minutes according to the literature (Lowe and Ruley 1993). According to literature the half-lives of the majority of the proteins that are regulated through protein turnover are less than 2 hours. Pro-caspase 3 and 7 were stable in IMR90 for 8 to 12 hours (data not shown). Same stability was observed for pro-caspase 3 and 7 in IMR90 expressing E1A. This indicates that pro-caspases are not regulated by E1A on the level of protein stability.

IMR90 expressing E1A have much higher levels of labeled pro-caspase 3 and 7 than IMR90 with control vector (Fig. 3C). That may indicate that the rate of protein synthesis is much higher in E1A-expressing cells. To examine protein synthesis rate IMR90 were short labeled for 20 minutes with [^{35}S]methionine. Labeled pro-caspase 3 and 7 were immunoprecipitated and theirs levels were compared between IMR90 infected with vector alone and with E1A (Fig. 3D). As

expected, protein synthesis rate for pro-caspase 3 and 7 was dramatically higher in E1A-expressing cells, pointing toward translational regulation of pro-caspase levels by E1A.

To further analyze the mechanism of translational regulation association of caspase mRNA with polysomes was measured by polysome gradients (Fig. 3E). If pro-caspase mRNA is more translationally active in IMR90/E1A than in IMR90 as the above results suggest, then a larger portion of the caspase mRNA present in IMR90/E1A should be associated with larger polysomes compared with IMR90. Cells were collected and lysed in the presence of cycloheximide and $MgCl_2$, which stabilize the association of ribosomes with mRNA. The lysates were sedimented through a linear sucrose gradient and fractions were collected. RNA was extracted from each fraction and analyzed for the presence of caspase 3 and 7 mRNAs by dot-blot hybridization with a ^{32}P-labelled human caspase 3 and 7 cDNA probes. Polysomes were found at the bottom of the gradient in fractions 7-11, while monosomes were found in fractions 2-5. Caspase 3 and 7 mRNA from IMR90 expressing E1A was associated with larger polysomes than was caspase 3 and 7 mRNA from IMR90 alone. In IMR90/E1A cells, 80% of the caspase 3 mRNA and 73% of the caspase 7 mRNA was found in fractions 7-11 containing high molecular weight polysomes, while in IMR90 alone 27% of the caspase 3 mRNA and 25% of the caspase 7 mRNA were found in these same fractions. As an internal control, the distribution of β-actin mRNA was compared and shown to be very similar in IMR90 and IMR90/E1A cells. Thus, E1A appear to induce pro-caspase levels by altering the level of protein synthesis.

E1A Induces Pro-caspase Levels in Primary Cells through Inactivation of Rb.

Mutations that disrupt apoptosis and chemosensitivity correlate precisely with E1A ability to bind p300/p400 or Rb family members (Samuelson and Lowe 1997). CR2 region of E1A was shown to be required for physical association between E1A and Rb-related proteins (Wang et al. 1993). Inactivation of Rb but not p107 or p130 is critical for ability of E1A to induce apoptosis (Samuelson and Lowe 1997). CR2 region of E1A is required for induction of the majority of pro-caspases (Fig. 2, data not shown). If CR2 induce pro-caspases by inactivating a single Rb protein, then the ΔCR2 mutant should behave like full-length E1A in cells lacking its major target. To test this hypothesis full length E1A, ΔN and ΔCR2 mutants were introduced into wild type and Rb-/- MEFs, and compared for their ability to induce pro-caspase 3 and 7 (Fig. 4A). E1A induced similar pro-caspase levels in both cell types. Thus, as expected, loss of Rb does not impair pro-caspase induction. Moreover in Rb-/- MEFs alone pro-caspase 3 and 7 levels were already elevated. As was anticipated ΔN mutant did not have any differences in ability to induced pro-caspase 3 and 7 levels irrespective of MEFs genotype. Concordant with previous results ΔCR2 was unable to induce levels of both pro-caspases in wild type MEFs. By contract, ΔCR2 mutant behaved like full length E1A in Rb-/- cells where pro-caspases were already induced. Thus, inactivation of Rb is a critical function of CR2 important for pro-caspases

induction. Furthermore, pro-caspase levels are elevated in cells lacking Rb. Thus, one of the multiple functions of Rb in the cells is to block pro-caspase induction.

To further investigate the requirement of Rb pathway for pro-caspase induction ability of E1A to induce pro-caspases in the absence of E2F1 transcription factor was measured (Fig. 4B). E2F1-/- MEFs expressing E1A showed similar levels of pro-caspases as wild type cells. Thus, E2F1 is not required for pro-caspase induction.

E1A induces apoptosis and chemosensitivity largely through induction of p53 through $p19^{ARF}$. To determine the requirement of p53 and ARF for pro-caspase induction E1A was introduced into p53-/- and ARF-/- MEFs and its ability to elevate pro-caspase levels in these cells was compared to wild type cells (Fig. 4C). E1A induced pro-caspases equally well in all cell types arguing that p53 or ARF are dispensable for pro-caspase induction.

Since overexpression of E2F1 induces apoptosis in primary MEFs (Qin et al. 1994; Shan and Lee 1994), ability of overexpressed E2F1 to induced pro-caspase levels was investigated. E2F1 was introduced into wild type MEFs (Fig. 4D) and E2F1-ER to IMR90 (Fig. 4E). Because E2F1-expressing MEFs rapidly underwent apoptosis to overcome the obstacle of getting enough cells for analysis E2F1 was also introduced to p53-/- MEFs which are resistant to apoptosis (Fig. 4D). Overexpression of E2F1 in MEFs and E2F1-ER in IMR90 after induction with tamoxifen was sufficient to increase pro-caspase 3 and 7 levels, raising the possibility that other E2F-factor than E2F1 is responsible for propagating the signal through Rb to increased translation rate of the pro-caspases.

Rb represses pro-caspases in human tumor cells.

Two human osteosarcoma cell lines with known Rb status were used to determine whether Rb is required for pro-caspase induction in these cell lines. E1A was introduced into U2OS - Rb-positive and into SAOS2 - Rb-deficient cells lines and level of pro-caspase 7 was compared (Fig. 5A). Concordant with results in primary cells, SAOS2 cells alone exhibit much higher levels of pro-caspase 7 than U2OS cells. E1A greatly increased level of pro-caspase 7 in U2OS cells and only slightly in SAOS2 cells. Thus, despite the fact that these two cell lines have a lot of other mutations theirs Rb – status is a sole indicator of the pro-caspase levels. Furthermore, the fact that SAOS2 cells already have elevated pro-caspase levels implies that one of the multiple functions of Rb in the cells is to block pro-caspase induction.

To prove that Rb acts as a suppressor for pro-caspases wild type and the tumor derived Δ22 Rb mutant were reintroduced into Rb-deficient SAOS2 cell line and their ability to suppress pro-caspase 3 and 7 levels were measured (Fig. 5B). As expected wild type Rb efficiently down regulated pro-caspase 3 and 7. In contrast, tumor-derived Δ22 Rb mutant that is known to be defective in most of Rb cell cycle regulating and tumor suppressing functions (Horowitz et al. 1990)

was unable to suppress pro-caspases. Thus, pro-caspase suppression is one of the many functions of Rb.

DISCUSSION

Understanding how oncogenes promote apoptosis and sensitivity to anticancer agents is pivotal for winning the war against cancer. Although Rb - E2F1 - p19^{ARF} - p53 pathway was implicated in oncogene-mediated signal transduction (Samuelson and Lowe 1997; Bates et al. 1998; de Stanchina et al. 1998; Kamijo et al. 1998) other pathways exits as well. Despite the latest advances in implicating APAF1-Caspase-9 pathway in mediating oncogene-dependent death signaling, the mechanisms by which this pathway becomes activated are still largely unknown (Fearnhead et al. 1998; Juin et al. 1999; Soengas et al. 1999). It was shown that cells expressing E1A showed increased levels of APAF1 and Caspase-9 pointing towards possibility of oncogenes regulation of cell death machinery at the level of gene expression (Fearnhead et al. 1998). In this study we took genetic and biochemical approach to investigate how apoptotic machinery is regulated by oncogene expression. We demonstrated that E1A oncogene upregulates expression of many pro-caspases in embryonic fibroblasts and in tumor cell lines through enhanced translation of caspases mRNAs. This pro-caspase induction strictly depends on E1A ability to deregulate Rb-E2F pathway and is p19^{ARF} and p53-independent.

E1A sensitizes cells to undergo apoptosis in part by increasing transcription of p19^{ARF} and stabilizing p53. Cells lacking p19^{ARF} and p53 or have low levels of p53 were shown to be resistant to apoptosis (de Stanchina et al. 1998). By analogy, our data shows that pro-caspases are induced by E1A but at the different level, the level of translation efficiency. Is this induction biologically significant as induction of p19^{ARF} and p53? It is not that trivial to prove that the absence of pro-caspases will attenuate oncogene-mediated apoptosis since the absence of knockout animal lacking all caspases genes or since it is not experimentally possible to decrease levels of all pro-caspases by immunodepletion to the levels in normal cells. Although there is correlative evidence that make us believe that pro-caspase induction by E1A is important for oncogene-mediated apoptosis. First, primary embryonic fibroblasts that have very low levels of pro-caspases are resistant to apoptosis triggered by multiple anticancer agents, but the same fibroblasts expressing E1A oncogene with 5 to 10 folds increase of pro-caspase levels are extremely sensitive (data not shown, (Lowe et al. 1993; McCurrach et al. 1997). Second, Rb-/- embryonic fibroblasts have 2 to 3 times more pro-caspases then wild type fibroblasts and they have increased sensitivity to anticancer agents (Almasan et al. 1995). Third, it was shown that wild type fibroblasts overexpressing E2F1 rapidly die by apoptosis (Qin et al. 1994; Shan and Lee 1994) and the same cells have induced pro-caspase levels. Though it is impossible to prove the requirement of pro-caspase induction for oncogene-dependent apoptotic sensitization of the cells it is suggestive that this induction may synergise with stabilization of p53 by E1A and with other E1A triggered events to promote chemosensitivity. c-myc oncogene

also sensitise cells to a variety of pro-apoptotic stimuli. This sensitization is mediated solely through release of cytochrome c from mitochondria (Juin et al. 1999). Unlike c-myc, E1A believe to exert its apoptotic activity not only through cytochrome c release but also downstream of it (Juin et al. 1999)[Fearnhead, 1998 #78]. We suggest that this downstream event could be the higher levels of pro-caspases in E1A-expressing cells, which help to accelerate apoptosis downstream of cytochrome c release.

Since caspases are synthesized in the cells in the form of inactive precursors (zymogens) which undergo rapid proteolytic processing during the early stages of apoptosis understanding the mechanism of this regulation is extremely valuable for our ability to control apoptosis in cancer cells. Caspase activation may occur by autoactivation, transactivation or proteolysis by other proteinases. Caspases zymogens have low but detectable proteolytic activity (Salvesen and Dixit 1999) suggesting that under certain conditions (clustering, high enough concentration, sufficient time) they can process themselves or other caspase zymogenes (Yamin et al. 1996; Muzio et al. 1998). Furthermore, overexpression of wild type pro-caspases but not their catalytically inactive mutants results in pro-caspase processing and activation, providing evidence that high enzyme concentration may facilitate autoprocessing (Orth et al. 1996). In vivo caspase recruitment to the signaling complexes through adapter molecules facilitates pro-caspase autoprocessing by bringing zymogenes close together, restricting their mobility, thereby increasing the local enzyme concentration. Forced oligomerization of pro-caspase-8 triggers pro-caspase processing and promotes apoptosis (Muzio et al. 1998). Once apical caspases are processed they can transactivate downstream caspases. Propagation of caspases cascade and final outcome of apoptotic response will thus depend on which caspases a cell expresses and the relative local concentrations of each caspase. Overexpression of caspases precursors in different cell type induces apoptosis (Miura et al. 1993; Fernandes-Alnemri et al. 1994; Kumar et al. 1994; Wang et al. 1994; Boldin et al. 1996; Srinivasula et al. 1996). Increased expression of pro-caspases genes thus might be an efficient way to increase local concentration of these zymogenes that will facilitate oligomerization and processing. Thus, increasing caspase levels may accelerate cell death and make it more efficient. Little is known about the factors that control expression of pro-caspases. Our data provides one of the first evidence that caspase regulation can occur at the level of pro-caspase expression. Oncogenes can promote apoptosis through mechanism involving upregulation of pro-caspases by increasing local concentration of these enzymes which facilitating their activation.

Our data argue that E1A ability to upregulate pro-caspases depends on altering Rb-E2F pathway. This pathway leading to p53 stabilization through $p19^{ARF}$ was already implicated in apoptotic sensitization of the cells by E1A (Samuelson and Lowe 1997; Bates et al. 1998; de Stanchina et al. 1998; Kamijo et al. 1998). Our data show that ability of E1A to induce pro-caspases levels is independent on p53 function providing the insight into p53-independent apoptosis. p53-independent apoptosis was shown to be important in some cell types (Strasser et al. 1994; Macleod et al. 1996) and can be triggered by E2F1 (Qin et al. 1994;

Hsieh et al. 1997; Phillips et al. 1997). Thus, both pathways (p53-dependent and independent) can originated from deregulation of Rb and may cooperate to promote efficient cell death. The fact that Rb can regulate caspases by repressing their protein levels provides the first connection between Rb, known cell cycle regulator, and cell death machinery. Our data together with the earlier observations that Rb is cleaved and inactivated during apoptosis by caspases (Janicke et al. 1996; Chen et al. 1997; Tan et al. 1997) provide an evidence for the existence of feedback loop to inactivate Rb during onset of apoptosis.

These studies describe a completely novel mechanism by which Rb through E2F's can influence gene expression. We show that Rb represses pro-caspases by altering their translation level. It is known that forced proliferation increases rate of mRNA translation (Brooks 1977). The rate-limiting factor of initiation of translation is binding of mRNA to the 40s ribosomal subunit. Several translation initiation factors participate in this event. One of them is eIF4E that binds to the mRNA cap site and possess an RNA helicase activity. Two phosphoproteins 4E-BP1 and 4E-BP2 involved in regulation of eIF4E activity. Dephosphorylated forms of 4E-BP's can bind directly to eIF4E and inhibit its translation-promoting activity (reviewed in (Brown and Schreiber 1996; Sonenberg 1996). It was shown that expression of E1A leads to hyperphosphorylation of 4E-BP's leading to activation of translation (Gingras and Sonenberg 1997). Although eIF4E is required for translation of all capped RNA, increased expression of eIF4E leads to a selective increase in translation of certain mRNA species (reviewed in Rhoads 1993; Mader and Sonenberg 1995). Our data suggest that E1A have a well-pronounced promoting effect on translation. But despite this overall effect, E1A increases translation of caspases mRNAs to greater extent then translation of β-actin mRNA which are translated very efficiently even without E1A presence. This observation points towards the existence of some sort of selectivity in ability of E1A to upregulate translation of specific genes. We suggest that eIF4E translation factor can be responsible for this selectivity.

KEY RESEARCH ACCOMPLISHMENTS

- E1A induces pro-caspases on the protein level
- This induction is p53 and p19 ^{ARF}-independent
- Caspase induction by E1A occurs mostly through deregulation of Rb pathway
- Caspase induction occurs in Rb-deficient fibroblasts in the absence of E1A and fibroblasts overexpressing E2F-1 transcription factor
- E1A increases pro-caspase 7 levels in a tumor cell line with intact Rb protein and reintroduction of Rb into an Rb-deficient tumor line reduces caspase levels
- E1A does not increase caspase gene transcription or protein stability
- E1A enhances the translation of pro-caspases mRNAs

REPORTABLE OUTCOMES

The manuscript of the above research with the title "E1A increases the translational efficiency of caspase mRNAs by inactivating Rb" is in preparation and will be submitted to PNAS this September.

Polyakova, J., Nahle, Z, Lee, T.H., Lazebnik, Y.A., Pelletier, J., Lowe, S.W. 2000 Induction of pro-caspases by E1A oncogene. Cancer genetics and tumor suppressor genes meeting at CSHL, p. 147

Ph.D. thesis is in preparation and will be defended in October of 2000.

CONCLUSIONS

See DISCUSSION section of Aim 4 in BODY.

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APPENDICES

FIGURE LEGENDS

FIGURE 1. E1A oncogene induces pro-caspase levels. (A) E1A induces pro-caspase levels in mouse embryonic fibroblasts (MEFs). (B) E1A induces pro-caspase levels in human fibroblasts (IMR90). E1A was introduced into populations of MEFs and IMR90 by retroviral mediated gene transfer. 30 μg of total cell lysates extracted with RIPA buffer from wild type MEFs and IMR90 infected with either vector alone or E1A construct were separated on 12% SDS-PAGE. Pro-caspase expression was examined by immunoblotting using mono- or polyclonal antibodies specific to mouse or human pro-caspases: caspase 1 (66441A, PharMingen); caspase 2 (13951A, PharMingen), caspase 3 (C31720 and C76920, Transduction labs), caspase 7 (1-1-10, Y. Lazebnik), caspase 8 (2-1-11), caspase 9 (AAP-119, Stressgen). E1A levels were determine by Western blot analysis with the M58 monoclonal antibodies. Expression of α-tubulin (B-5-1-2, Sigma) is shown as a loading control.

FIGURE 2. Functional mapping of E1A regions required for pro-caspase induction. Pro-caspase expression in populations of MEFs (A) and IMR90 (B) expressing the empty vector, full-length E1A and two E1A deletion mutants ΔN (deletion of amino acids 2-36) and ΔCR2 (deletion of amino acids 120-140) was examined by immunoblotting with anti-caspase 3 (C76920 and C31720, Transduction labs) and 7 (1-1-10, Y.Lazebnik) antibodies. E1A levels were

determine by Western blot analysis with the M58 monoclonal antibodies. Expression of α-tubulin (clone B-5-1-2, Sigma) is shown as a loading control.

FIGURE 3. E1A mediated pro-caspase induction occurs at the level of protein translation. (A) Northern blot analysis of caspase mRNA expression in MEFs. Total RNA was prepared from MEFs infected with either the E1A-expressing virus or control virus using ULTRASPEC RNA isolation system (Biotecx). 10 μg was loaded per lane on agarose formaldehyde gel. Northern blots were performed according manufactures instructions (Boehringer Mannheim) using cDNA probes for mouse caspases. cDNA fragments were labeled by random priming (Hexanucleotide mix, Boehringer Mannheim). 18S RNA was used as a loading control. (B) Role of proteosome proteolysis in regulation of pro-caspase turnover. Two known specific proteosome inhibitors – peptide aldehydes (LLnL and MG132) were used to treat MEFs at final concentration of 40 mM in DMSO for various amounts of times (0 to 24 hours). Calpain 2 inhibitor LLM that is incapable of inhibiting proteosome was used as a control. 30 μg of total cell lysates extracted with RIPA buffer were separated on 12% SDS-PAGE. Pro-caspase 3 and 7 were detected by Western blot. p53 Western was performed as a control for known accumulation of p53 protein (CM5, Novocastra). (C) Pulse-chase analysis of pro-caspase stability in E1A-expressing cells. IMR 90 expressing empty vector or full length E1A were incubated for 1 hours with medium lacking methionine, labeled for 1 hour with 400 μCi/ml of ^{35}S-labeled amino acids, washed twice with PBS, and chased with 2mM of unlabeled methionine and cysteine. Cells were collected at various times, and caspase 3 and 7 was immunoprecipitated out of total cell extract (normalized by CPM counts) using C31720 (Transduction labs) and 1-1-10 (Y.Lazebnik) antibodies respectively. Immunoprecipitated samples were resolved on 12% SDS-PAGE, gels were dried and subjected to radioautography. Stability of non-specific background protein to anti-caspase 7 antibodies shown as a control. (D) Translation efficiency rate analysis. IMR 90 expressing empty vector or full length E1A were incubated for 1 hour with medium lacking methionine and short labeled for 20 minutes with 400 μCi/ml of ^{35}S-labeled amino acids. Cells were collected and caspase 3 and 7 was immunoprecipitated out of total cell extract (normalized by CPM counts) using C31720 (Transduction labs) and 1-1-10 (Y.Lazebnik) antibodies respectively. Immunoprecipitated samples were resolved on 12% SDS-PAGE, gels were dried and subjected to radioautography. Total cell protein labeled with [^{35}S] Methionine without immunoprecipitation (normalized by CPM counts) was used as a control. (E) Association of caspase mRNA with polysomes. The association of caspase 3 and 7 mRNA with polysomes in IMR90 infected with control virus and E1A-expressing virus was compared. Cell extracts containing polysomes were prepared and loaded on a 15-50% linear sucrose gradient. Eleven fractions were collected. RNA was extracted from each fraction by RNAeasy Mini kit (Qiagen) and the amount of caspase 3 and 7 mRNA in each fraction was determine by dot-blot hybridization analysis with ^{32}P-labeled human caspase 3 and 7 cDNA probe. β-actin probe was used as a control. 18S probe served as a control for distribution of rRNA

distribution of rRNA alone polysome gradient. The positions of ribosomal subunits (monosomes) and polysomes are indicated.

FIGURE 4. Genetics of Rb-dependent pro-caspase suppression in primary MEFs and in IMR90. (A) Deletion of CR2 region of E1A does not prevent pro-caspase induction in Rb-/- MEFs. Expression of pro-caspase 3 and 7 was compared in wild type versus Rb-/- MEFs expressing either vector or full-length E1A or two E1A deletion mutants ΔN and ΔCR2. (B) Rb-dependent pro-caspase suppression does not require E2F1 transcription factor. Ability of E1A to induce pro-caspase 3 and 7 levels was compared in wild type, Rb-/- and E2F1-/- MEFs by Western blot analysis. (C) Rb-dependent pro-caspase suppression does not depend on p53 and ARF. Ability of E1A to induce pro-caspase 3 and 7 levels was compared in wild type, p53-/- and ARF-/- MEFs by Western blot analysis. (D) Overexpression of E2F1 in primary MEFs induces pro-caspase 3 and 7 levels. Full length E1A, E1A mutants and E2F1 were introduced into populations of wild type, Rb-/-, E2F1-/-, p53-/- and ARF-/- MEFs by retroviral mediated gene transfer. 30 μg of total cell lysates were separated on 12% SDS-PAGE. Pro-caspase expression was examined by immunoblotting with mouse specific anti-caspase 3 and 7 (1-1-10, Y.Lazebnik) antibodies. (E) Overexpression of E2F1 in IMR90 induces pro-caspase 3 and 7 levels. E2F1-ER was introduced into IMR90 by retrovirus. Infected cells were incubated in DMEM medium (Gibco) containing 200 nM 4-hydroxytamoxifen (4-OHT) (Sigma) for 0, 4, 8,12,and 24 hours. After 4-OHT treatment, cells were harvested and 30 μg of total cell lysates were separated on 12% SDS-PAGE. Pro-caspase expression was examined by immunoblotting with human specific anti-caspase 3 (C31720, Transduction labs) and 7 (1-1-10, Y.Lazebnik) antibodies. E2F1 levels were determine by Western blot analysis with the E51120 antibodies (Transduction laboratories). p53 Western was performed as a control for known induction of p53 protein (CM1, Novocastra). Expression of α-tubulin (B-5-1-2, Sigma) is shown as a loading control.

FIGURE 5. Genetics of Rb-dependent pro-caspase suppression in tumor cell lines. (A) Rb suppresses pro-caspase 7 in osteosarcoma cell line U2OS. Two osteosarcoma cell lines with known Rb status U2OS (Rb-positive) and SAOS2 (Rb-negative) expressing either vector alone or E1A were compared for pro-caspase 7 protein expression levels. (B) Reintroduction of wild type Rb into Rb-deficient SAOS2 cell line suppresses pro-caspase 3 and 7 levels. Wild type Rb and Δ22 deletion mutant of Rb were introduced into SAOS2 cell line together with GFP vector by transient transfection. Two days after transfection cells were sorted and positive for GFP cells were collected and used for Western blot analysis. 30 μg of cell lysates were separated on 12% SDS-PAGE. Pro-caspase expression was examined by immunoblotting with human specific anti-caspase 3 (C31720, Transduction labs) and 7 (1-1-10, Y.Lazebnik) antibodies. Rb expression levels were determined by Western blot analysis with the 14001A antibodies (PharMingen). Expression of α-tubulin (clone B-5-1-2, Sigma) is shown as a loading control.

See attached figures # 1 through 5.

Figure 1. E1A oncogene induces pro-caspase levels.

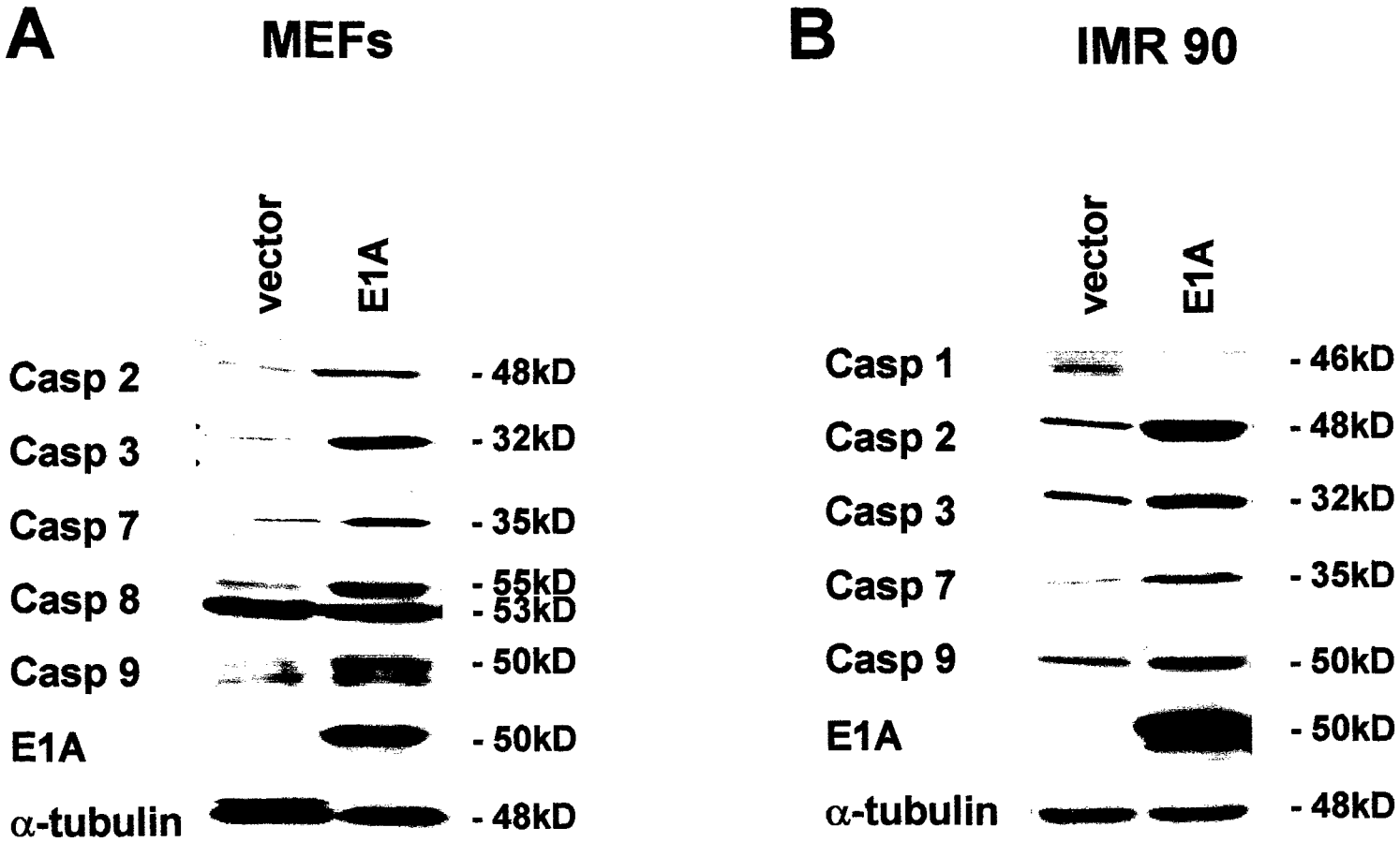


Figure 2. Functional mapping of E1A regions required for pro-caspase induction.

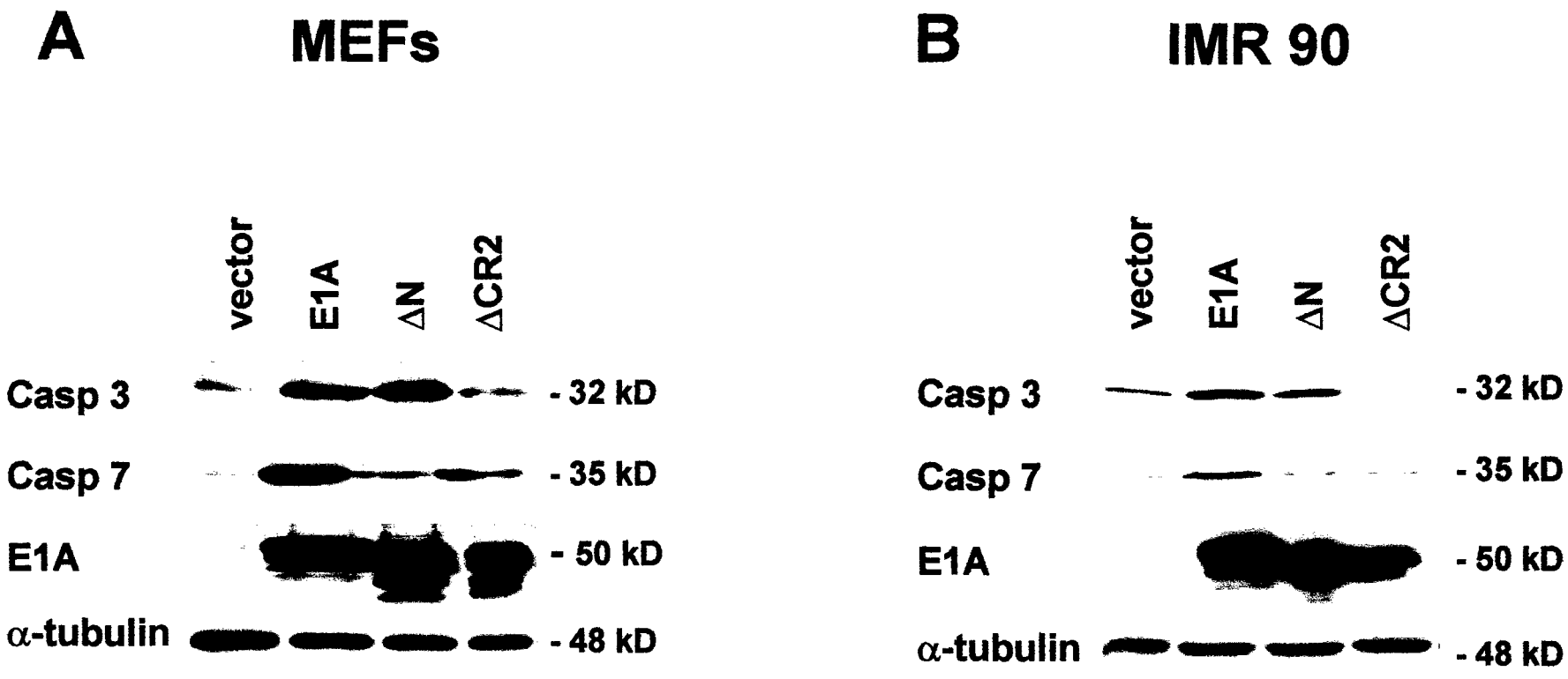
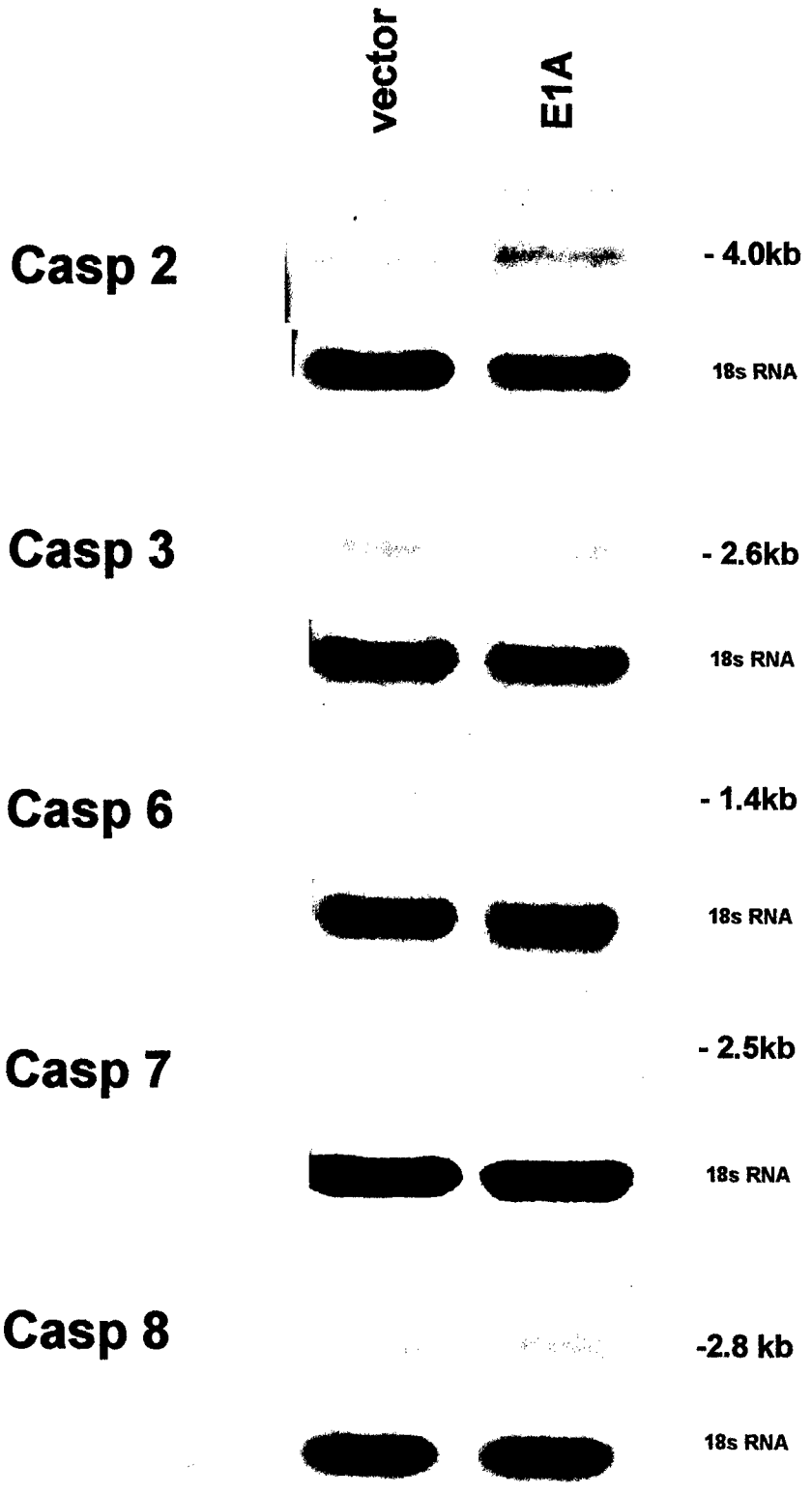
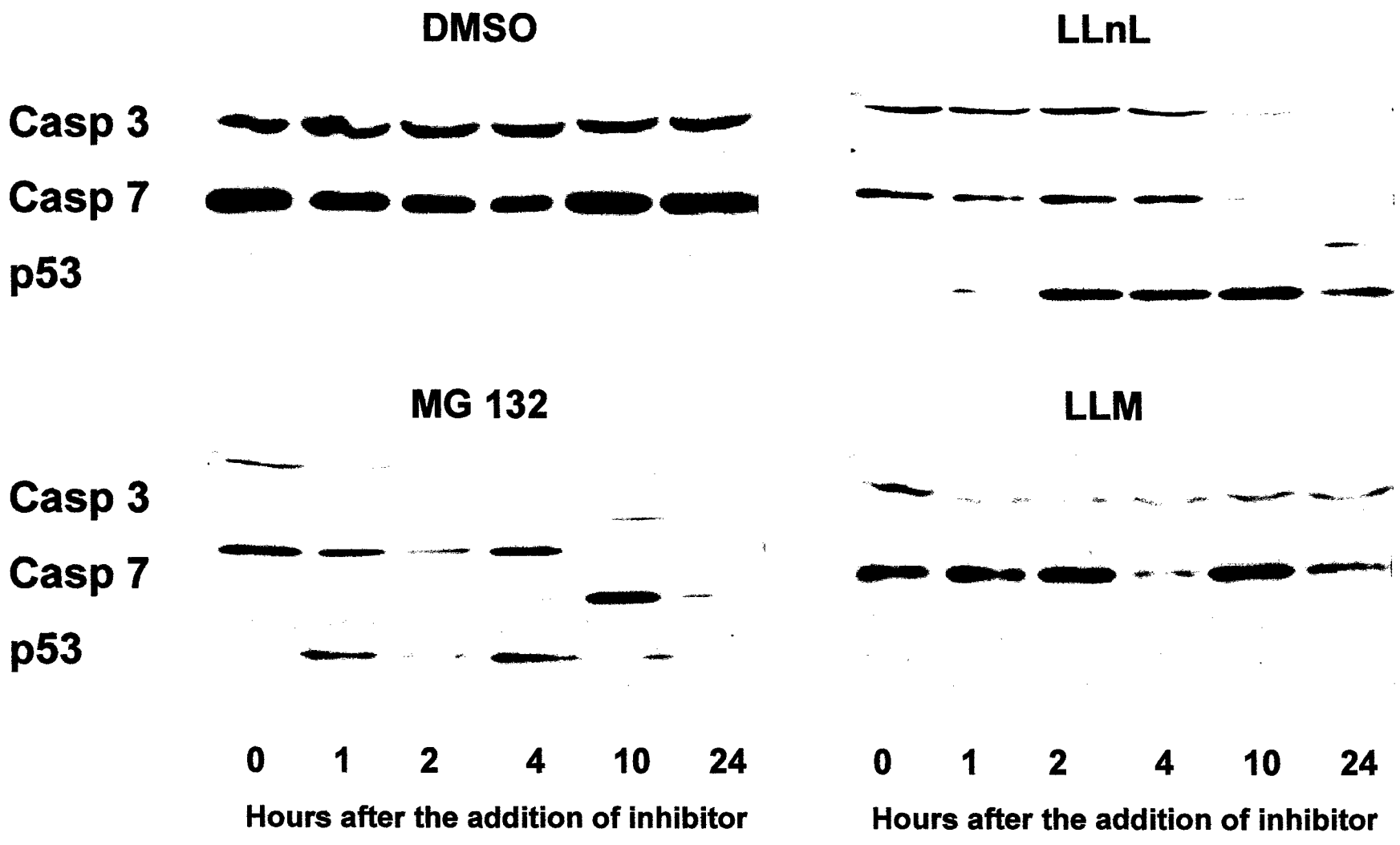


Figure 3. E1A mediated pro-caspase induction occurs at the level of protein translation.

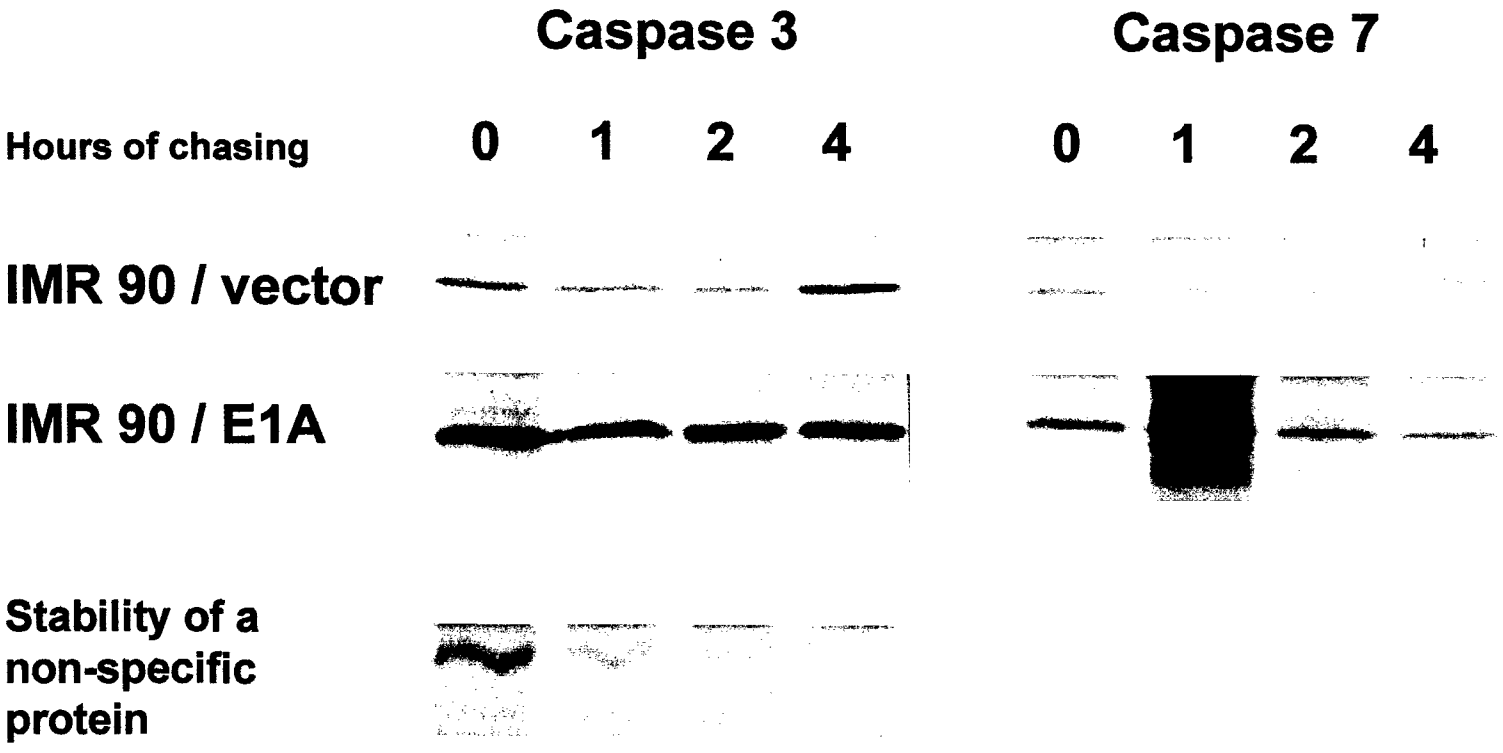
A


vector
E1A
Casp 2
- 4.0kb
18s RNA
Casp 3
- 2.6kb
18s RNA
Casp 6
- 1.4kb
18s RNA
Casp 7
- 2.5kb
18s RNA
Casp 8
-2.8 kb
18s RNA

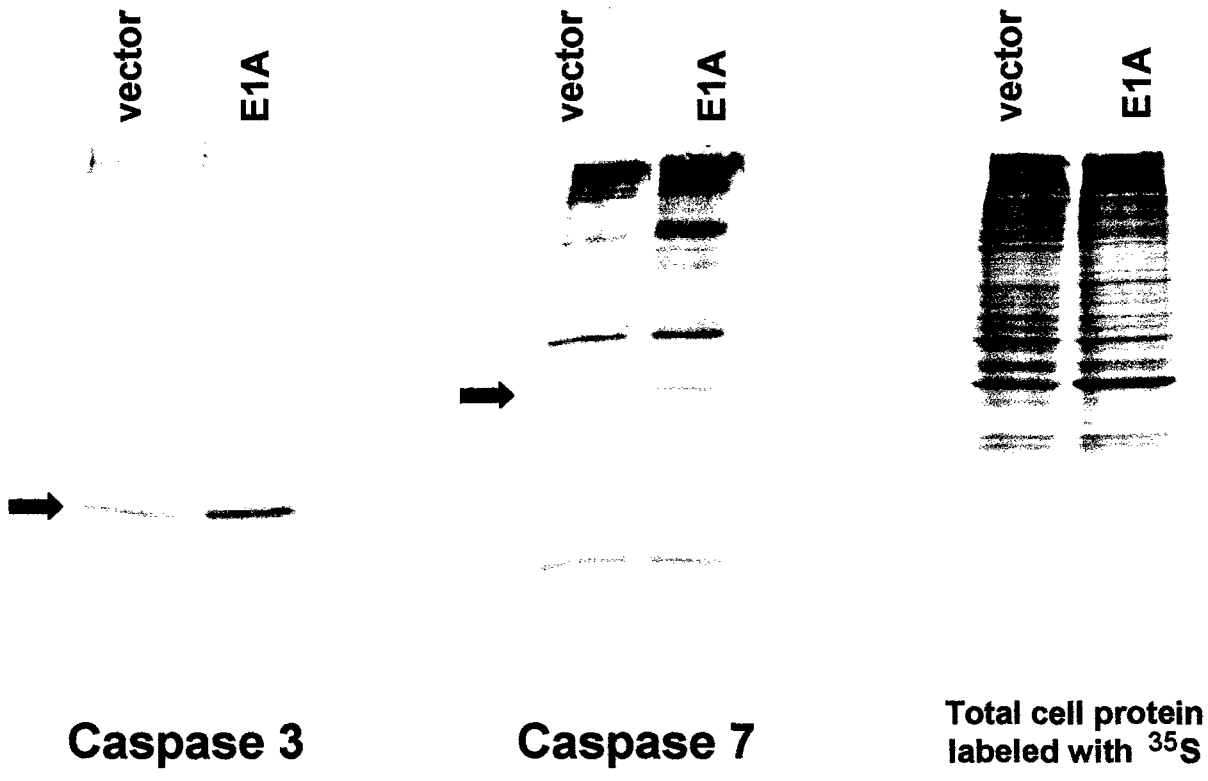
B


DMSO
LLnL
Casp 3
Casp 7
p53
MG 132
LLM
Casp 3
Casp 7
p53
0 1 2 4 10 24
Hours after the addition of inhibitor
0 1 2 4 10 24
Hours after the addition of inhibitor

C


Caspase 3
Caspase 7
Hours of chasing
0 1 2 4
0 1 2 4
IMR 90 / vector
IMR 90 / E1A
Stability of a non-specific protein

D

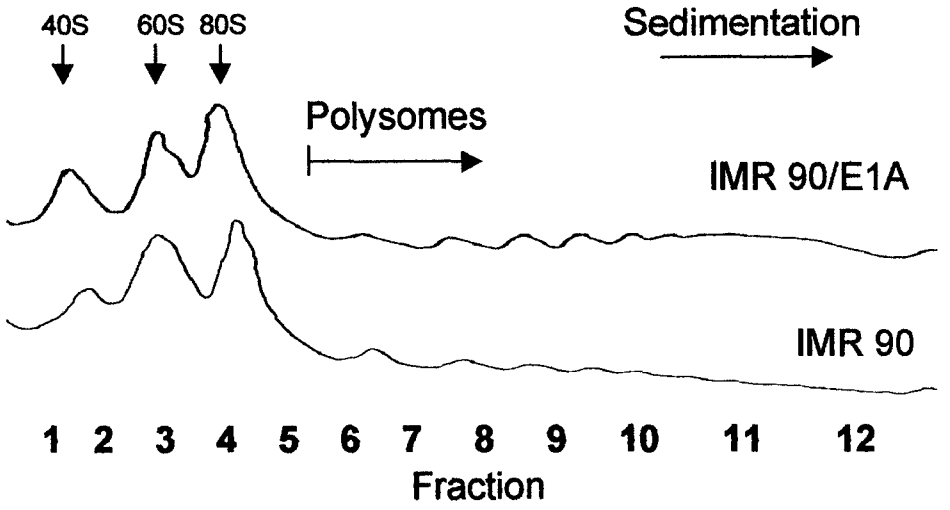


Caspase 3

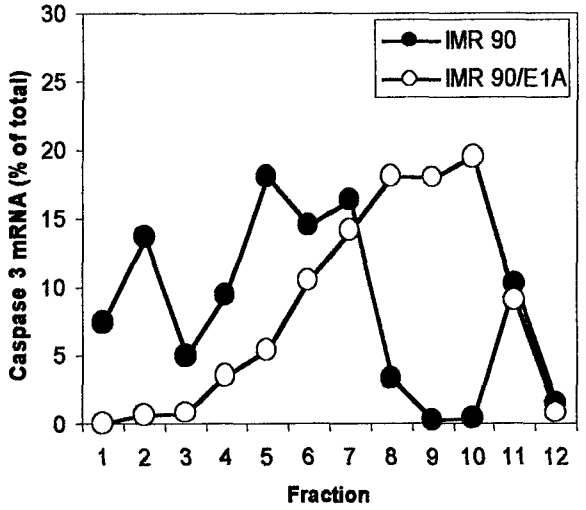
Caspase 7

Total cell protein labeled with ^{35}S

E



Caspase-3 mRNA



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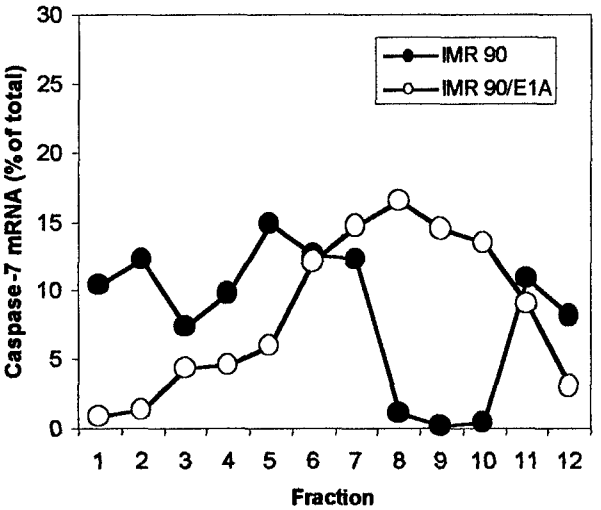
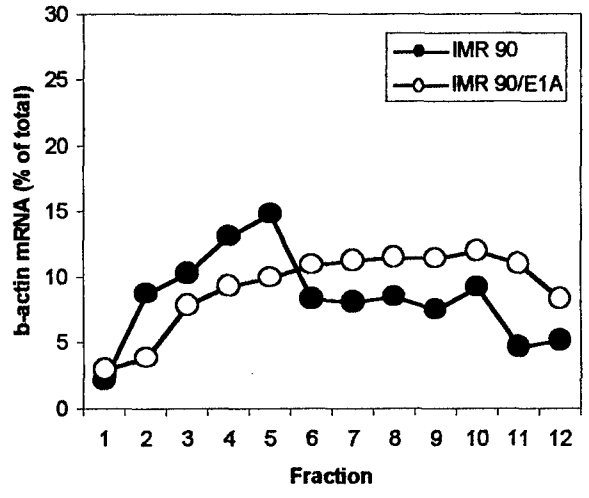


β-actin mRNA



18s mRNA

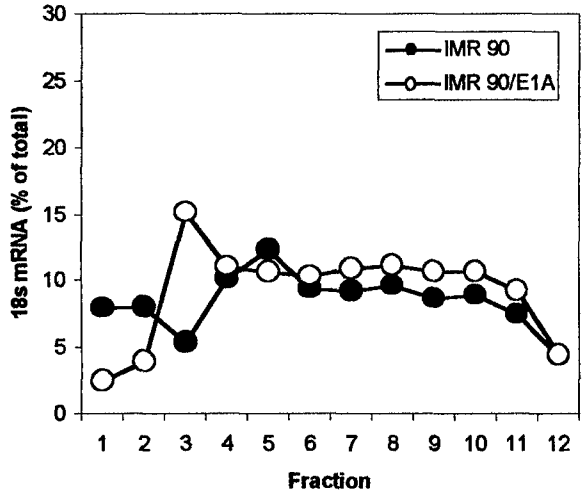
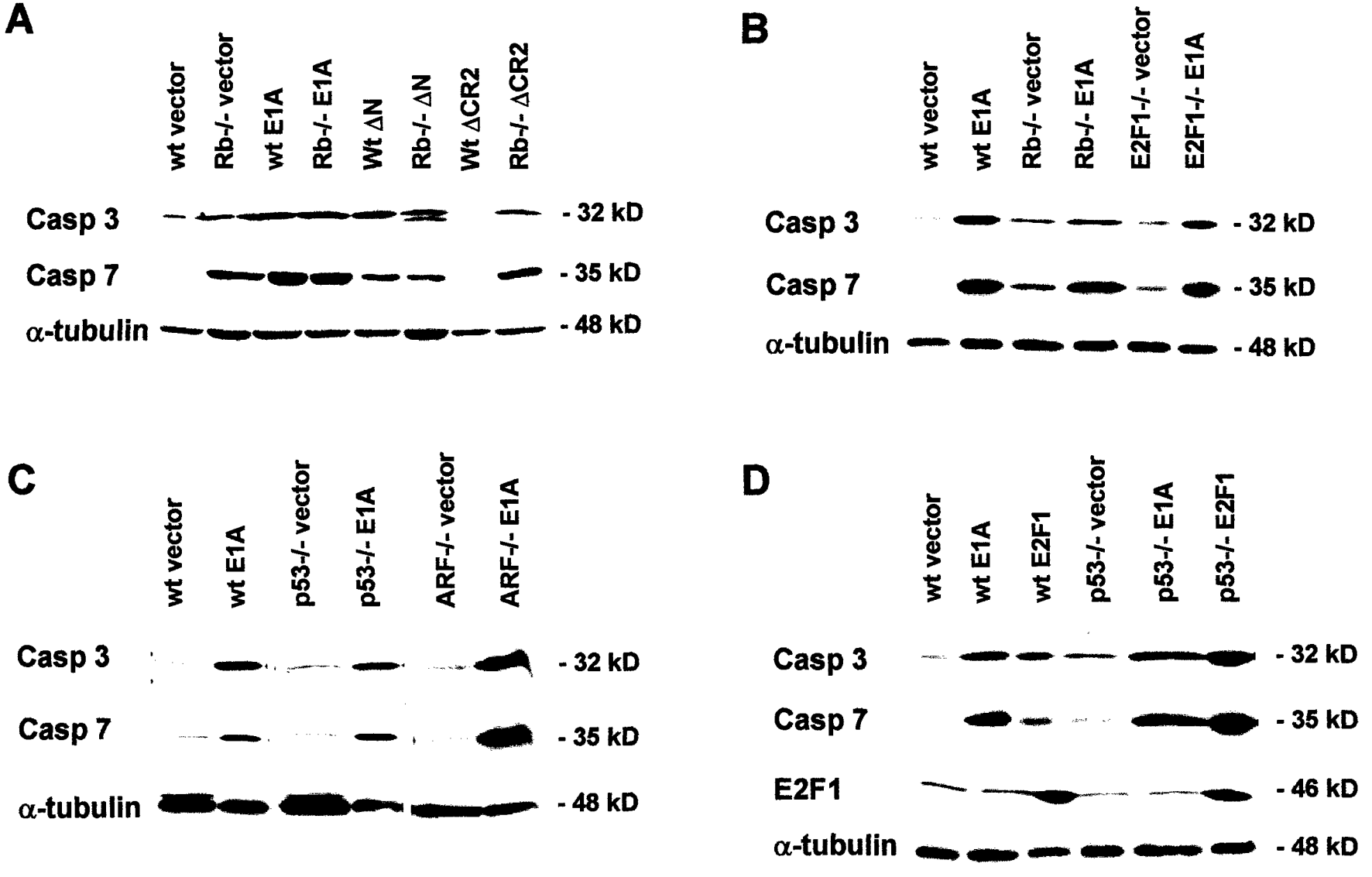


Figure 4. Genetics of Rb-dependent pro-caspase suppression in primary MEFs



E

IMR 90

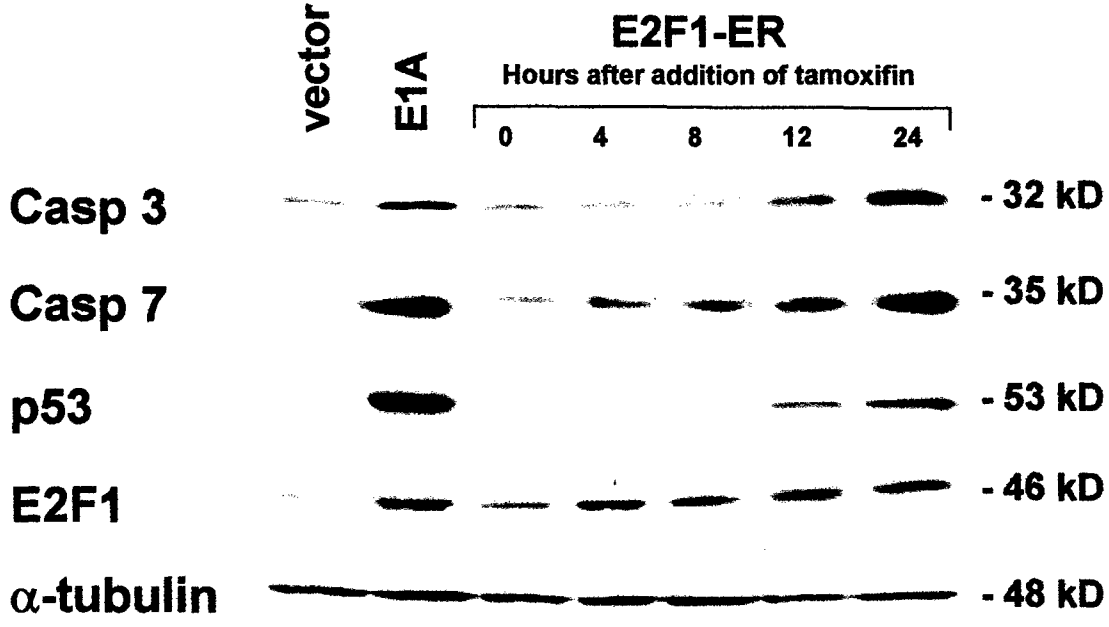

vector
E1A
E2F1-ER
Hours after addition of tamoxifin
0
4
8
12
24
Casp 3
Casp 7
p53
E2F1
α-tubulin
- 32 kD
- 35 kD
- 53 kD
- 46 kD
- 48 kD

Figure 5. Genetics of Rb-dependent pro-caspase suppression in tumor cell lines